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Sensitive quantification of roflumilast and roflumilast N-oxide in human plasma by LC–MS/MS employing parallel chromatography and electrospray ionisation

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ABSTRACT

A high throughput bioanalytical method based on semi-automated liquid extraction and liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been developed for the sensitive quantification of roflumilast and its metabolite roflumilast N-oxide, a phosphodiesterase (PDE) inhibitor in human plasma and serum. The sample work-up procedure comprised liquid extraction using penta-deuterated analogues of both analytes as internal standards. Chromatography was performed on C18 revered phase analytical columns at a flow rate of 0.5 mL/min in the dual column mode employing a column switching technique and a linear gradient from 18% to 54% acetonitrile in 0.005 M aqueous ammonium acetate containing 0.006% formic acid. Mass spectrometry was performed on an API 4000 instrument in the positive ion SRM-mode (selected reaction monitoring) with the Turbo-V® ionspray interface. The method showed linear detector responses over the entire calibration range between 0.1 ng/mL (lower limit of quantification (LLOQ)) and 50 ng/mL (upper limit of quantification (ULOQ)) for both analytes. Linear regression analysis with concentration-squared weighting ($1/x^2$ for roflumilast and $1/x$ for roflumilast N-oxide) yielded inaccuracy and precision values <15% and coefficients of correlation (r) for the calibration curves >0.99 for both analytes.

1. Introduction

Daxas®, Roflumilast [(**I**), 3-cyclopropylmethoxy-4-difluoromethoxy-N-(3,5-dichloropyridyl-4)-benzamide], is a selective inhibitor of phosphodiesterase 4 (PDE4). It was registered in the EU in 2010 and launched on the US market in 2011 for the treatment of severe chronic obstructive pulmonary disease (COPD) which is among the world's most prevalent diseases [1–3]. Daxas® is also currently at the regulatory approval phase in other countries. The pharmacological properties of roflumilast and its mode of action have been characterised in human cells and in preclinical models of COPD and asthma. PDE4 inhibitors interfere with the breakdown of cAMP which leads to an increase in the intracellular concentration of cAMP. This, in turn, activates protein kinase A (PKA), which catalyses the phosphorylation of proteins, leading subsequently to a reduction of inflammation. Thus, roflumilast provided evidence in numerous preclinical pharmacology models to exert its potent anti-inflammatory activity by targeting neutrophils, eosinophils, T-lymphocytes, macrophages and dendritic cells [4,5]. The main metabolite of roflumilast in humans and in most animal species is roflumilast N-oxide, which is pharmacodynamically active and adds considerably to the overall biological activity of the parent compound [6]. The metabolite is formed by phase I metabolism of roflumilast with CYP3A4 and CYP1A2 as being the predominantly involved enzymes and a minor contribution of CYP2C19 and CYP1A1.

The earlier developed conventional quantitative high-performance liquid chromatography (HPLC) assays with classical ultraviolet or fluorescence detection for roflomilast and the metabolite (results not published) provided insufficient sensitivity and robustness in the lower ng/mL range. Additionally, this method comprised long chromatographic run times, complex post-column derivatisation and a labour intensive work-up procedure which nowadays are not favoured to analytically support large clinical pharmacokinetic studies.

HPLC with tandem mass spectrometry (MS–MS) using pneumatically assisted electrospray ionisation (ESI) in combination with parallel chromatography (dual column switching) and semi-automated liquid/liquid extraction should provide the required sensitivity, straightforward sample work-up, and fast capability for trace level quantification of roflumilast and its metabolite in biological matrices after oral administration of roflumilast in the dose range of 500 μg.

As a result, the development of a sensitive assay which enables the quantification in the lower ng/mL range of both analytes, roflumilast (**I**) and its metabolite roflumilast N-oxide (**II**) had to be envisaged.

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In order to enable clinical investigators to individually adjust (or terminate) the dosing therapeutic drug monitoring on a 'standby' basis is often required by clinical studies units at an early stage of a study in cases of potential inter-individual variabilities in bioavailabilities of the drug in combination with undesired side-effects.

Our aim was to develop a fast mass spectrometric method to analyse roflumilast and its metabolite in human plasma samples in the low ng/mL range during ongoing pharmacokinetic studies using semi-automated liquid/liquid extraction and parallel chromatography with Turbo-V®-ESI-MS/MS. In order to increase the sample-throughput and enable us to report plasma concentrations to the clinical pharmacology unit as fast as possible we envisaged to reduce the run time per sample by implementing a dual analytical column chromatography approach. Therefore, a 'column-switching' technique was chosen to be linked with triple quadrupole mass spectrometric detection.

2. Experimental

2.1. Materials

Roflumilast, roflumilast N-oxide, [$^2H_5$]-roflumilast and [$^2H_5$]-roflumilast N-oxide were provided from Nycomed GmbH (former ALTANA Pharma AG, Konstanz, Germany). Isotopic purities of [$^2H_5$]-roflumilast and [$^2H_5$]-roflumilast N-oxide were greater 99% for both compounds, respectively.

Acetonitrile (HPLC grade), ammonium acetate (pro analysi.), dimethyl sulfoxide (pro analysi.), ethyl acetate (HPLC grade), formic acid (pro analysi.) methanol (HPLC grade), *n*-heptane (pro analysi) were purchased from Merck KgaA (Darmstadt, Germany). Deionised water (HPLC grade) was generated within Nycomed GmbH by a Millipore MilliQ®-system (Schwalbach, Germany). Blank human plasma was generated within the clinical unit of Nycomed GmbH.

An ammonium acetate working solution (0.005 M) was prepared by dilution of 385.4 mg ammonium acetate to 1000 mL deionised water and adjusting the pH to 5.0 by adding formic acid (0.006%, v/v) (mobile phase A). Mobile phase B consisted of 90% aqueous acetonitrile (v/v) containing 0.005 M ammonium acetate (i.e. 385.4 mg ammonium acetate in 1000 mL of mobile phase B). High purity nitrogen for the mass spectrometer was provided from a central boil-off tank (Sauerstoffwerk Friedrichshafen GmbH, Friedrichshafen, Germany).

2.2. Instrumentation

Sample analysis was performed by using liquid chromatography with pneumatically assisted electrospray in combination with tandem mass spectrometric detection (LC–ESI-MS/MS). The LC–MS/MS system consisted of an AB Sciex API 4000 triple quadrupole mass spectrometer (Toronto, Canada) equipped with a Turbo-V® ionspray source operation in the positive ESI mode. Two Agilent 1100 binary HPLC-pumps and degasser-systems (Agilent, Böblingen, Germany) and an CTC HTS PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) fitted with a 100 μL-Hamilton syringe (Chromtech, Idstein, Germany) feeding two injection ports comprising 50 μL sample-loops each. The autosampler was equipped with a cooling stack for storing the samples during analyses at approximately +10 °C. A Tecan Genesis Freedom 200 robot (8-channel-system) (Tecan Group Ltd., Männedorf, Switzerland) was used for pipetting and semi-automated liquid/liquid extraction of the plasma samples in the 96-well plate format. For solvent evaporation, a Turbo Vap 96-system was utilised (Zymark, Idstein, Germany). Centrifuges were from Eppendorf Zentrifugen GmbH (Leipzig, Germany) (Centrifuge 5810R) and from Heraeus GmbH (Multifuge 4KR) (Hanau, Germany).

2.3. Liquid chromatographic conditions

Chromatographic separation of the analytes was carried out on a Luna Phenomenex® C18(2), 5 μm particle size, 100 Å, 50 mm (length) × 2 mm (I.D.) reversed phase analytical column with an in-line stainless steel frit from Thermo Fisher Scientific (Bonn, Germany). A linear gradient was employed at a flow rate of 500 μL/min. The autosampler temperature was set at +10 °C. After injection of the sample (t = 0 min) the mobile phase composition was kept for 1 min at 18% mobile phase B. Within the following 2 min the content of the mobile phase was raised to 54% of mobile phase B followed by an increase to 100% of mobile phase B for another minute. A drop in the mobile phase composition to 20% of mobile phase B was maintained for a time period of 1 min for re-equilibration purposes of the column to the gradient starting conditions. The run-time per single column was 5 min. The injection volume of the samples was 20 μL. The retention times for roflumilast/[$^2H_5$]-roflumilast and roflumilast N-oxide/[$^2H_5$]-roflumilast N-oxide were 3.9 min and 3.7 min, respectively. For parallel chromatography in a dual column-switching mode the CTC PAL autosampler was equipped with two separate 6-port injection valves feeding two identical analytical columns that were connected with the mass spectrometer via a third Rheodyne-6-port valve (Rheodyne Europe GmbH, Alsbach a.d. Bergstrasse, Germany). From t = 0 to t = 2.5 min the third Rheodyne-6-port valve was used to truncate non-relevant signals arising from column 1 into waste. At t = 2.5 min injection of the next sample onto column 2 was executed again diverting the LC-flow for the first 2.5 min into waste. Thus, an overall run-time per sample of 2.5 min was achieved.

2.4. Mass spectrometric conditions and data acquisition

The mass spectrometer was operated in the positive ion mode with to Turbo-V® ionspray source performing pneumatically assisted electrospray at a voltage of +4200 V at 600 °C. The nebulizer gas (GS1) was set to 40 psi, heater gas (GS2) to 70 psi and curtain gas to 40 psi. Collision gas thickness was set to 6.00×10^{15} molecules/cm^3. The position of the electrospray capillary was adjusted to 5 mm horizontal and 5 mm vertical. The entrance potential (EP) was maintained at +10.0 V. The declustering potential (DP), collision energy (CE) and the cell exit potential (CXP) were set at +91, +39 and +14 V for roflumilast, +96, +39 and +14 V for roflumilast N-oxide, respectively. For the corresponding internal standards the declustering potential (DP), collision energy (CE) and the cell exit potential (CXP) were set at +96, +41 and +14 V for [$^2H_5$]-roflumilast, +91, +41 and +14 V for [$^2H_5$]-roflumilast N-oxide, respectively.

Selective reaction monitoring (SRM) was applied for quantification using the following mass transitions: for roflumilast and roflumilast N-oxide at m/z 403 → m/z 187 and at m/z 419 → m/z 187, respectively, employing dwell-times of 160 ms each; for the internal standards, [$^2H_5$]-roflumilast and [$^2H_5$]-roflumilast N-oxide, mass transitions were at m/z 408 → m/z 190 and at m/z 424 → m/z 190, respectively, employing dwell-times of 40 ms each. The triple quadrupole mass spectrometer was set up to work at unit mass resolution. The roflumilast and roflumilast N-oxide analysis data were acquired and quantified using the Analyst Software version 1.4.1 (AB Sciex, Toronto, Canada). Calibration curves were generated by plotting the peak area ratios of the analytes and internal standards against their theoretical concentrations. A least square regression with concentration-squared ($1/x^2$) weighting was employed to calculate the calibration curves for roflumilast,

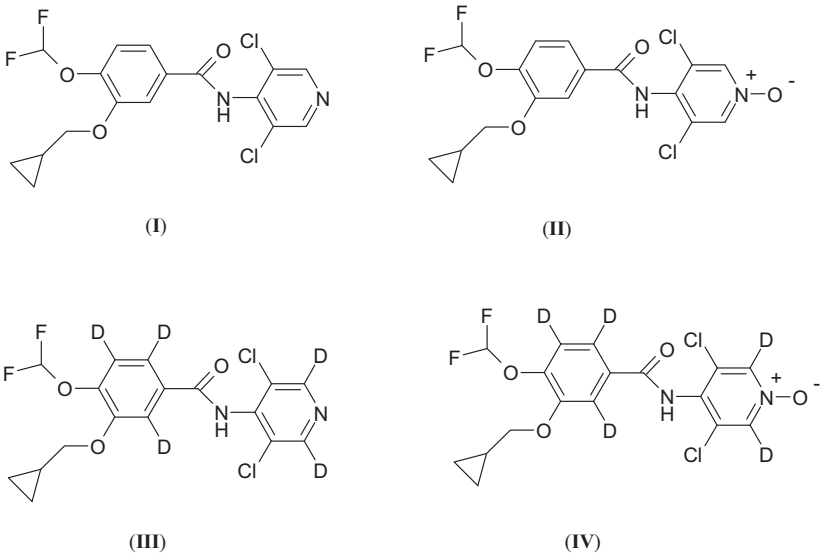

Fig. 1. Chemical structures of roflumilast (**I**), the major metabolite (**II**) and the corresponding penta-deuterated internal standards (**III**, **IV**).

and a regression with concentration ($1/x$) weighting was employed to calculate the calibration curves for roflumilast N-oxide.

2.5. Standard and sample preparation

Separate stock solutions of roflumilast and roflumilast N-oxide (free bases) were prepared in methanol at 300 μg/mL. Plasma working dilutions containing both analytes (roflumilast and roflumilast N-oxide) were generated from these stock solutions at 8000 ng/mL. Subsequently, from the 8000 ng/mL a further working solution in plasma was prepared at 800 ng/mL by 10-fold dilution. Additionally, a 20 ng/mL plasma working solution was prepared by 40-fold dilution of the 600 ng/mL working solution. With these three plasma working solutions the final concentrations of the calibrators at 0.1, 0.25, 0.75, 2.5, 5.0, 10.0, 25.0 and 50 ng/mL were generated by dilution with the appropriate volumes of blank plasma.

For the internal standards, [$^2H_5$]-roflumilast and [$^2H_5$]-roflumilast N-oxide, separate stock solutions were prepared in methanol at 500 μg/mL. A combined intermediate dilution in methanol was prepared from these stock solutions, containing both internal standards, at concentrations of 10,000 ng/mL and 5000 ng/mL for [$^2H_5$]-roflumilast and [$^2H_5$]-roflumilast N-oxide, respectively. Subsequent 1000-fold dilution with blank plasma yielded a combined internal standard working solution at 10 ng/mL and 5 ng/mL for [$^2H_5$]-roflumilast and [$^2H_5$]-roflumilast N-oxide. All plasma solutions were stored frozen at approximately −20 °C except the methanolic stock and working solutions of the analytes which were kept at approximately +4 °C.

For the quality control (QC) samples separate stock solutions were prepared in methanol at 300 μg/mL. A combined intermediate dilution in plasma was prepared from these stock solutions, containing both analytes at 6000 ng/mL. Subsequent dilution with plasma yielded the following QC samples QC-high (40 ng/mL), QC-medium (7.5 ng/mL) and QC-low (0.3 ng/mL). QC samples were stored frozen at approximately −20 °C in 300 μL aliquots. Stability of thus frozen QC samples is guaranteed for at least 36 months.

2.6. Protocol for sample extraction

200 μL of plasma sample (double blank samples, blank samples, calibrators, QC samples, study samples) were added to 50 μL of internal standard solution containing both ISTDs (10 μg/mL [$^2H_5$]-roflumilast and 5 μg/mL [$^2H_5$]-roflumilast N-oxide in plasma) in a 96-well deep-well block. Subsequently, 600 μL of ethyl acetate/*n*-heptane (50/50, v/v) were pipetted into each well by a liquid handling robot. After sealing the deep-well block, vigorous automated mixing (5 min) was followed by centrifugation (5 min at approx. 3000 rpm). 500 μL of the (upper) organic layer were transferred to a second deep-well plate. After evaporation under a stream of nitrogen the residue was reconstituted with 15 μL of DMSO and 25 μL of water. After centrifugation (5 min, 3000 × *g*) of the deep well block, 20 μL were injected into a 50 μL-sample loop for HPLC–MS/MS analysis.

2.7. Quantification

Calibration curves were constructed by plotting peak area ratios of the analytes and the internal standards against the analytes' concentrations. The results of the raw data were evaluated with the AB Sciex quantification software (Analyst® 1.4.1). Analyst® was used to calculate the weighted linear regression fit of the peak areas of the standards of [$^2H_5$]-roflumilast and [$^2H_5$]-roflumilast N-oxide (calibrators) relative to their corresponding internal standards. The weighted linear regression line ($1/x^2$ for roflumilast and $1/x$ for roflumilast N-oxide) was fitted over the 500-fold concentration range. It was found that the weighting by the concentration-squared ($1/x^2$) factor yielded the best accuracies for the back-calculated values of the QC samples in comparison to concentration ($1/x$), response ($1/y$) or response-squared ($1/y^2$) weighting for roflumilast. It was also found that the weighting by the concentration ($1/x$) factor yielded the best accuracies for the back-calculated values of the QC samples in comparison to concentration ($1/x^2$), response ($1/y$) or response-squared ($1/y^2$) weighting for roflumilast N-oxide. Drug and metabolite concentrations in unknown and quality control samples were calculated from these lines.

3. Results and discussion

3.1. Mass spectrometry

The lack of sufficient sensitivity and selectivity of the previously developed HPLC assay with fluorescence detection after post-column photochemical derivatisation in combination with labour intensive work-up procedures was the driving force for us to

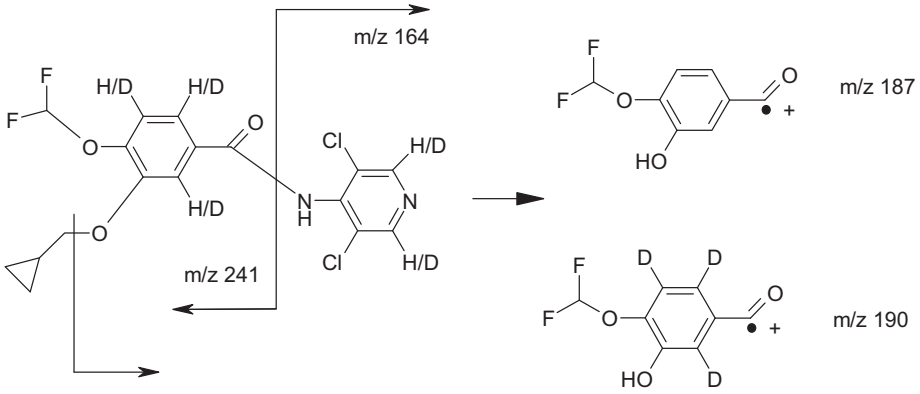

Fig. 2. Proposed fragmentation and postulated major product ions used for quantification formed from roflumilast and [$^2H_5$]-roflumilast.

develop an HPLC–MS/MS assay with a simple, straight-forward and semi-automated liquid–liquid extraction in a 96-well-plate format.

Electrospray ionisation sources have proven to be one of the most commonly used and highly universal interface in bioanalytical mass spectrometry [7]. In particular pneumatically assisted electrospray in the Turbo-V® mode provides enormous advantages for quantitative biopharmaceutical determinations of high polar and low molecular mass compounds [8–11].

The structural formulas of the analytes and their corresponding internal standards are displayed in Fig. 1. Two main fragments can be observed by α-cleavages for roflumilast (Fig. 2). Fig. 3 shows the pneumatically assisted electrospray positive product ion mass spectrum of roflumilast using the protonated molecular ion (m/z 403) as the precursor ion. The first (minor abundant) fragment ion at m/z 241 is produced by a loss of 162 u from the protonated molecular ion representing a cleavage (neutral loss) of the 3,5-dichloro-pyridyl-amino moiety. The most abundant fragment is formed by a cleavage of both, the 3,5-dichloropyridyl-amino group and the cleavage of the 3-cyclopropylmethylene group. Thus, the postulated radical cation at m/z 187 is formed, which is used for the quantification representing the transition of m/z 403 to m/z 187. The mass spectrometer's parameters (e.g. capillary and lens voltages given in the 'tuning-file') were set such to optimise the abundance of the major fragment ion at m/z 187 and subsequently yielding the low limit of quantification for roflumilast. In addition, the adjustment of the declustering potential controlling the

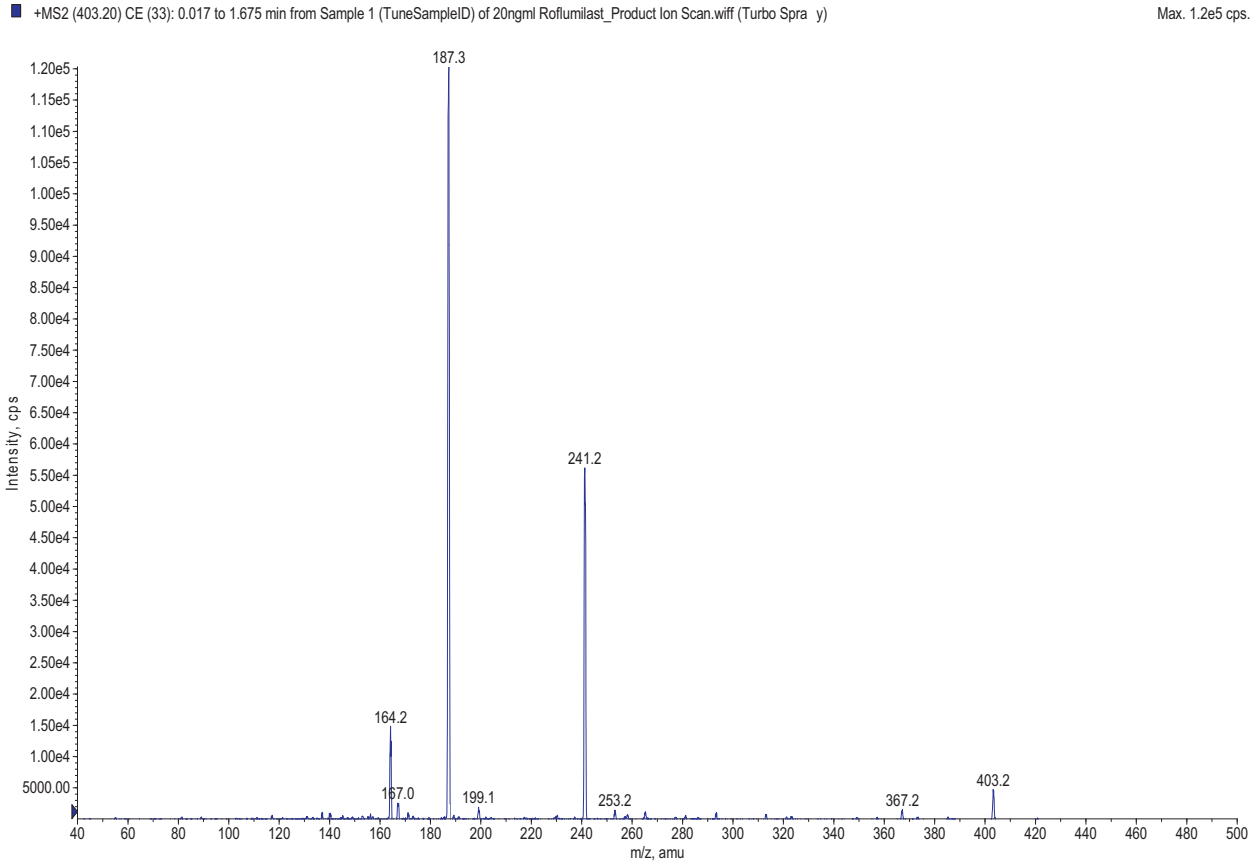

Fig. 3. Positive product ion mass spectrum of roflumilast (**I**) precursor ion at m/z 403; ~20 pg/μL at a flow rate of 10 μL/min presented to the TurboV® (ESI-LC–MS/MS mode) ion source via infusion.

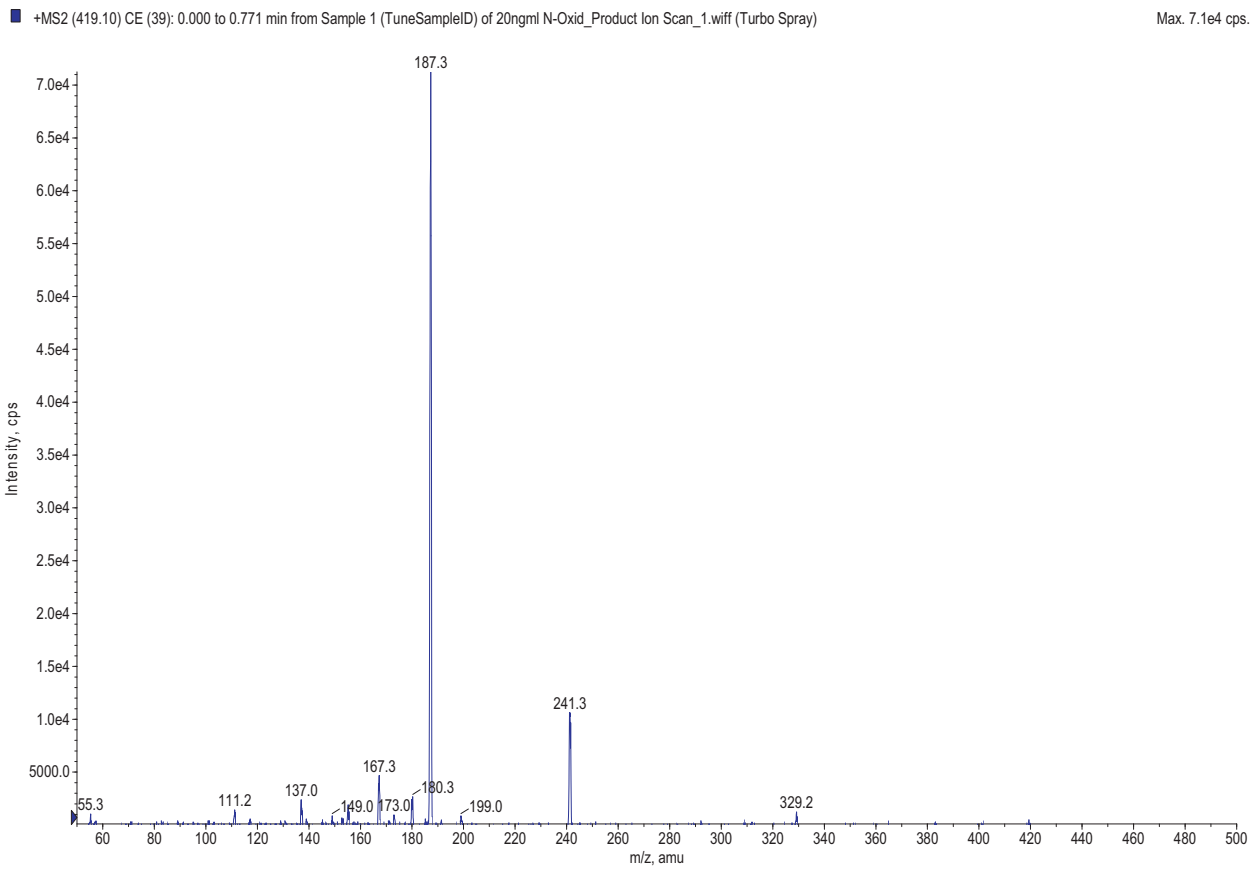

Fig. 4. Positive product ion mass spectrum of the major metabolite, roflumilast N-oxide (**II**), precursor ion at m/z 419; ~20 pg/μL at a flow rate of 10 μL/min presented to the TurboV® (ESI-LC–MS/MS mode) ion source via infusion.

'up-front fragmentation' voltage was set to achieve the best compromise of reducing of lower molecular weight clusters and maximum sensitivity for the precursor ions. Thus, an optimum yield of the pseudo-molecular ion ($[M+H]^+$: m/z 403) of roflumilast was allowed to enter the region of the first mass analyser (Q1). As for roflumilast, the metabolite (roflumilast-N-oxide) shows two major fragment ions at the same nominal m/z-values derived from the protonated molecular ion at m/z 419 (Fig. 4). Therefore, based on the obtained spectrum we postulate the identical structure for both the first (minor abundant) fragment ion at m/z 241 and the second (base peak) fragment ion at m/z 187 as for the roflumilast fragment ions. Therefore, the base peak fragment ions at m/z 187 were used for the quantification for both roflumilast and roflumilast-N-oxide. Whereas the minor abundant ions at m/z 241 could be used as 'qualifier' ion traces in cases were ambiguous chromatograms should occur (traces not displayed). Additional support for the postulated fragment ions of roflumilast and roflumilast-N-oxide is given by the fragmentation pattern of their penta deuterated internal standards ($[^2H_5]$-roflumilast and $[^2H_5]$-roflumilast-N-oxide (Fig. 1) in Fig. 2. The first (minor abundant) fragment ion at m/z 244 is produced by a loss of 164 u from the protonated molecular ion representing a cleavage (neutral loss) of the 3,5-dichloro-pyridyl-amino moiety. The most abundant fragment is formed by a cleavage of both, the $[^2H_2]$-3,5-dichloropyridyl-amino group and the cleavage of the 3-cyclopropylmethylene group. Thus, the postulated radical cation at m/z 190 is formed, which is used for the quantification representing the transition of the internal standard (for roflumilast) of m/z 408 to m/z 190. In a similar fashion as for the internal standard for roflumilast, the penta deuterated internal standard ($[^2H_5]$-roflumilast-N-oxide) for the metabolite shows two major fragment ions at the same nominal m/z-values (m/z 244, m/z 190) derived from the protonated molecular ion at m/z 424. Optimising of the mass spectrometer's parameters regarding the optimum fragmentation and formation of the analytical product ions in order to gain maximum sensitivity was performed for both analytes by automated tuning via the Analyst® software. The such obtained MS settings were then subsequently accepted for the mass spectrometric analysis of roflumilast and roflumilast-N-oxide in combination with their corresponding penta deuterated internal standards in the selected reaction monitoring (SRM) mode. The such taken approach in tuning the mass spectrometer proved to be suitable for the quantification of roflumilast and the metabolite since negligible mass "carry-over" or "cross-talk" from the plasma matrix (i.e. salts and proteins) or the internal standards was observed, when operating in the SRM mode. In order to proof this negligible mass "cross-talk", a typical product ion chromatogram of a

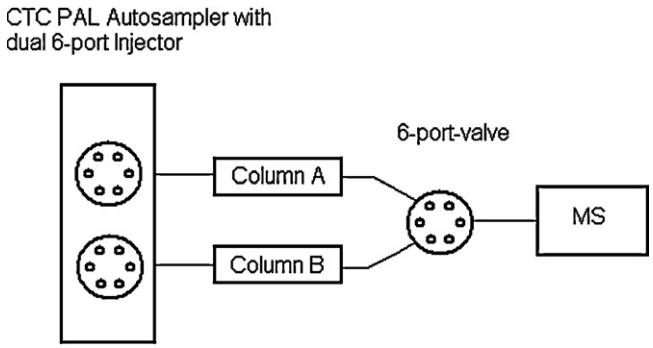

Fig. 5. Setup for parallel chromatography in the dual column mode.

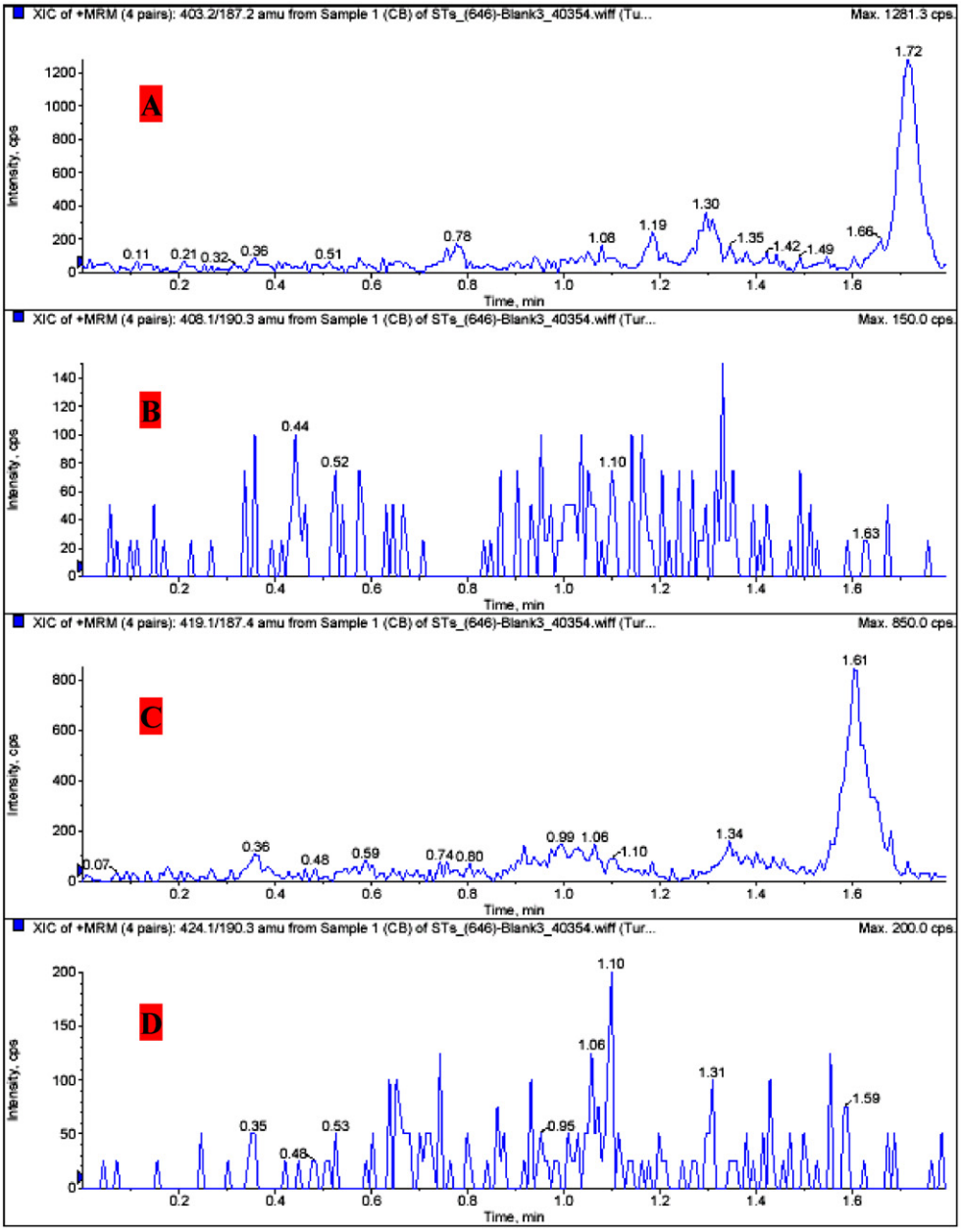

Fig. 6. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of roflumilast [channel A], the corresponding penta-deuterated internal standard [$^2H_5$]-roflumilast [channel B] and the metabolite [channel C] with the corresponding penta-deuterated internal standard [channel D: [$^2H_5$]-roflumilast N-oxide] in plasma extracts of a 'double blank' plasma sample. Expected retention times were at approximately 0.9 and 1.2 min.

"double blank" plasma sample is depicted and discussed in Section 3.2 (Fig. 6), illustrating the mass contributions for all four ion-transitions of roflumilast [channel A: m/z 403 → 187], its internal standard, [$^2H_5$]-roflumilast, channel B: m/z 408 → 190, the metabolite [channel C: m/z 419 → 187], and the metabolite's internal standard, [$^2H_5$]-roflumilast-N-oxide [m/z 424 → 190].

3.2. Chromatography

In order to develop a robust assay with high sample throughput and to achieve maximum sensitivity for the analytes we required as short a retention times as possible in combination with a rugged sample clean-up and chromatographic separation. The great advantage of having analytes with different molecular masses plus the availability of stable labelled internal standards as well as similar retention times in combination with tandem mass spectrometric detection, enabled us to aim at a minimum separation that removes salts and matrix components that can suppress and/or interfere with the analyses from the target components, while maintaining good sample throughput.

The need for effective sample clean up in combination with sound chromatographic separation in order to minimise matrix effects in quantitative LC–MS/MS analyses was also reported by many authors to affect the ionisation in pneumatically assisted electrospray mode to a significantly higher extent in comparison to the atmospheric pressure chemical ionisation [12–15].

In order to establish a high sample throughput LC–MS/MS assay to support pharmacokinetic studies with high numbers of plasma samples we decided to choose a semi-automated off-line liquid–liquid extraction with ethyl acetate/*n*-heptane (50/50, v/v) in the 96-well format using an 8-channel Tecan pipetting robot. A subsequent sample concentration step was implemented by

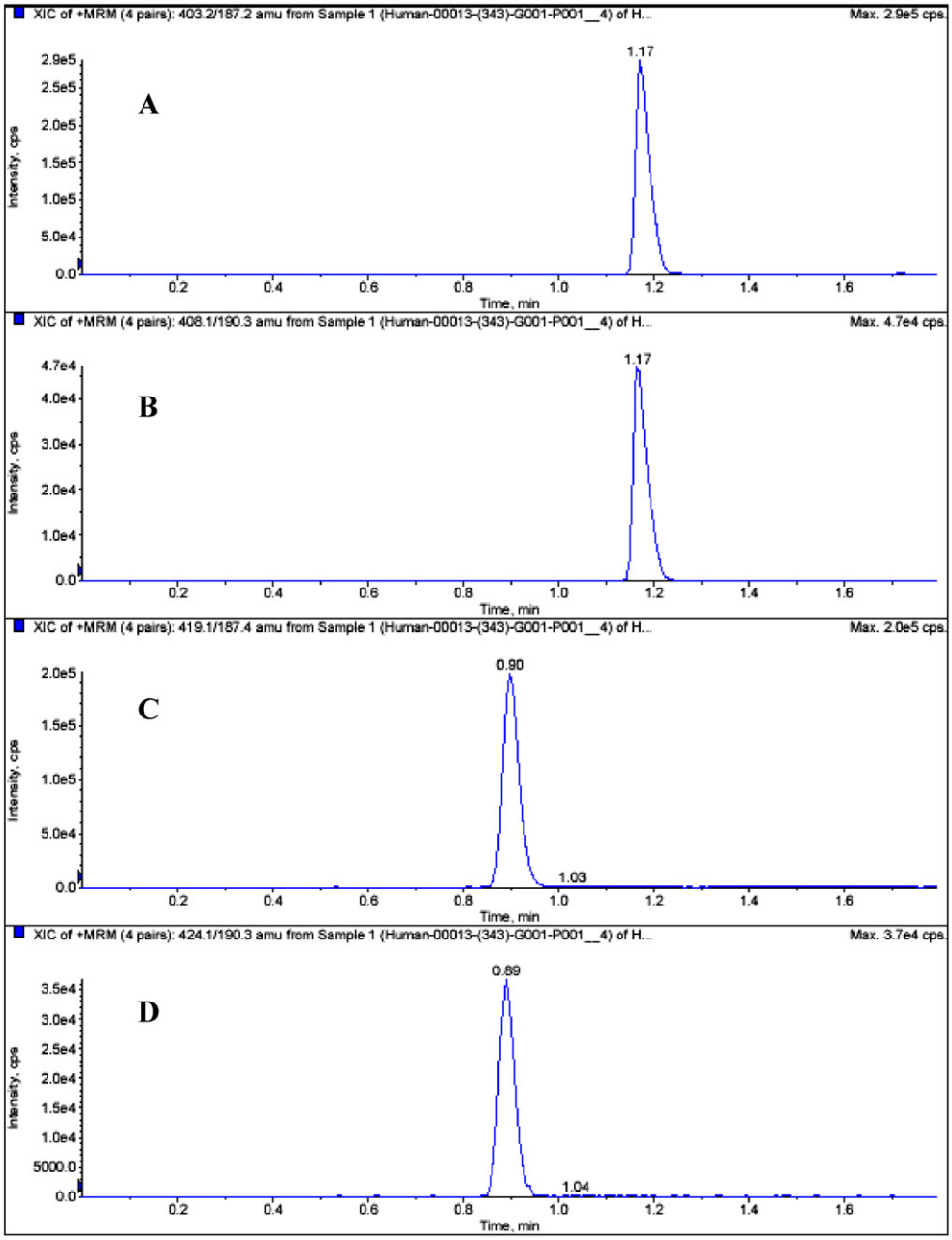

Fig. 7. Typical chromatograms obtained by selected reaction monitoring (SRM) during the determination of roflumilast [channel A], the corresponding penta-deuterated internal standard [channel B]: [$^2H_5$]-roflumilast, the metabolite [channel C] and the corresponding penta-deuterated internal standard [channel D]: [$^2H_5$]-roflumilast N-oxide (the internal standards' concentrations of [$^2H_5$]-roflumilast are at 10 ng/mL and of [$^2H_5$]-roflumilast N-oxide at 5 ng/mL, respectively). Measured concentration of roflumilast are at 9.86 ng/mL and of roflumilast N-oxide at 7.01 ng/mL, respectively. Plasma extract of a blood sample taken from a healthy male volunteer approx. 1 h after oral administration of 500 μg roflumilast.

reconstituting the evaporate in 40 μL aqueous DMSO. We achieved the best compromise regarding recovery and autosampler-stability for the analytes by using 15 μL DMSO and 25 μL water as reconstitution solution. Half of the thus obtained reconstitute was submitted for LC–MS/MS analysis. For chromatographic separation we established a parallel chromatography setup in a dual column-switching mode depicted in Fig. 5. This "column-switching" system consists of three 6-port Rheodyne switching valves. The CTC PAL autosampler contains two separate 6-port injection valves feeding two identical separate analytical columns (columns A and B). These analytical columns are linked with the third 6-port injection valve which connects the system to the mass spectrometer. In the first step an aliquot of 20 μL is injected onto column A at a mobile phase B composition of 18% for 1 min. Within the following 2 min the content of the mobile phase was raised to 54% of phase B, followed by an increase to 100% of the mobile phase B (90% aqueous acetonitrile containing 0.005 M ammonium acetate) at a flow rate of 0.5 mL/min using a linear gradient. Subsequently a drop to a mobile phase composition of 20% B was maintained for 1 min so that the column could re-equilibrate to gradient starting conditions. In order to avoid very polar, early eluting components entering the mass spectrometer's interface the third 6-port injection valve is set to divert the eluent flow from $t = 0$ to $t = 2.5$ min into waste. Simultaneously at $t = 2.5$ min the next sample is injected onto column B. As a result of employing two alternatingly loaded analytical columns in the described off-set mode the theoretical cycle-time per sample of

Table 1
Inter-assay performance for the determination of roflumilast (**I**).

Nominal concentration [ng/mL]	*n*	Calculated concentration [ng/mL]	Accuracy [%]	RSD [%]
(a) Inter-assay (between-day) performance of roflumilast standards (calibrators)				
0.1	6	0.1004	100.4	8.02
0.25	6	0.248	99.2	6.09
0.75	6	0.752	100.3	1.46
2.5	6	2.44	97.4	2.13
5.0	6	5.03	100.6	2.37
10	6	10.0	99.6	2.16
25	6	24.7	98.7	2.49
50	6	51.9	103.8	1.71
			Mean accuracy: 100.0	Mean RSD: ±3.30
(b) Inter-assay (between-day) performance of QC samples				
0.1	16	0.0995	99.5	8.88
0.3	18	0.296	98.7	6.85
7.5	18	7.39	98.6	2.17
40	18	39.6	99.0	2.64
			Mean accuracy: 98.95	Mean RSD: ±5.14

5 min in a single-column mode could be reduced to approximately 2.5 min and subsequently double the sample throughput.

After testing many of the vast variety of analytical columns available on the market, we obtained the best results regarding chromatographic separation, secession from plasma matrix components, and finally relatively short retention times with the Luna Phenomenex® C18(2), 5 μm particle size, 100 Å, 50 mm (length) × 2 mm (I.D.) reversed phase analytical column equipped with an in-line pre-column frit from Thermo. This reversed phase column provided us with the best compromise regarding reproducibility of chromatographic shape, and separation, as well as flow rate, high sample throughput and last not least cost effectiveness. All experiments to achieve acceptable chromatographic peaks on other reversed phase columns produced less satisfactory results. In order to achieve optimum mass spectrometric sensitivity and fragmentation for the analytes we decided to keep the HPLC eluent composition (Fig. 6 for results of a 'double blank' plasma sample). Hence, this compromise of sensitivity and chromatographic peak shape was accepted and no further attempts were made to further improve the chromatographic appearance such as 'peak-tailing' at the bottom end of the calibration curve (Fig. 7). The in-line pre-column frits were exchanged prior to every analytical run (approximately after 400 plasma sample injections). Furthermore, it turned out during routine analysis that one analytical column could be used with this set-up for analysis of at least 1000 plasma samples without deterioration such as decreasing signals and 'peak-tailing'.

A flow rate of 0.5 mL/min using a 50 mm × 2 mm (I.D.) column with the given fast linear gradient in combination with the pneumatically assisted electrospray interface provided the best compromise in terms of mass spectrometric detectability (i.e. sensitivity), chromatographic performance, and analytical ruggedness for the roflumilast and roflumilast N-oxide bioanalysis.

The advantage of the availability of stable labelled forms of roflumilast and roflumilast N-oxide as internal standards provided (nearly) identical physico chemical properties in chromatographic and mass spectrometric behaviour in terms of lipohilicity and recovery from the biological matrix after liquid/liquid extraction. The recoveries were assessed at approximately 100% for roflumilast and at approximately 85% for roflumilast N-oxide, respectively. Recoveries of I and II were determined by comparing peak areas and heights of post-extraction-spiked samples versus prior-extraction-spiked samples.

Roflumilast, the N-oxide metabolite, and the internal standards eluted at retention times of approximately 1.17 and 0.9 min, respectively. The cycle time per sample was 2.5 min. The great gain of this parallel chromatographic setup using three 6-port injection valves yielded in halving the 5 min cycle time compared to single column approach and is displayed in Fig. 5.

In order to illustrate the high selectivity of tandem mass spectrometric detection in SRM mode, selected ion chromatograms of

Table 2
Inter-assay performance for the determination of the metabolite (**II**).

Nominal concentration [ng/mL]	*n*	Calculated concentration [ng/mL]	Accuracy [%]	RSD [%]
(a) Inter-assay (between-day) performance of metabolite standards (calibrators)				
0.1	6	0.0992	92.2	12.65
0.25	6	0.256	102.3	5.26
0.75	6	0.745	99.4	3.50
2.5	6	2.46	98.4	3.43
5.0	6	4.99	99.8	1.31
10	6	9.89	98.9	1.06
25	6	24.97	99.9	2.46
50	6	51.01	102.0	1.76
			Mean accuracy: 99.1	Mean RSD: ±3.93
(b) Inter-assay (between-day) performance of QC samples				
0.1	17	0.104	103.7	8.72
0.3	18	0.293	97.8	4.48
7.5	18	7.42	98.9	1.92
40	18	39.4	98.4	2.62
			Mean accuracy: 99.7	Mean RSD: ±5.14

Table 3
Intra-assay (within-day) performance for the determination of roflumilast (**I**) in QC samples.

Nominal concentration [ng/mL]	*n*	Calculated concentration [ng/mL]	Accuracy [%]	RSD [%]
0.1	5	0.096	96.1	5.88
0.3	6	0.287	95.6	6.30
7.5	6	7.282	97.1	1.76
40	6	39.166	97.9	1.69
			Mean accuracy: 96.7	Mean RSD: ±3.91

Table 4
Intra-assay (within-day) performance for the determination of the metabolite (**II**) in QC samples.

Nominal concentration [ng/mL]	*n*	Calculated concentration [ng/mL]	Accuracy [%]	RSD [%]
0.1	5	0.097	97.5	13.05
0.3	6	0.293	97.5	2.20
7.5	6	7.483	99.8	0.78
40	6	40.110	100.3	1.82
			Mean accuracy: 98.8	Mean RSD: ±4.46

a "double blank" plasma sample after liquid/liquid extraction are given in Fig. 6. As a result, only negligible contributions occur at the retention times of roflumilast (1.2 min) and roflumilast N-oxide (0.9 min), and the internal standards. Selected ion chromatograms of a plasma extract from a blood sample taken 1 h after oral administration of 500 μg of roflumilast to a healthy volunteer are shown in Fig. 7. At these and at higher concentrations (>50 ng/mL) of roflumilast (channel A) and roflumilast N-oxide (channel C) in study samples we observed no matrix related peaks at retention times of 1.17 and 0.9 min at the channels of roflumilast (m/z 403 → 187), the metabolite (m/z 419 → 187), and both corresponding internal standards (m/z 408 → 190 and m/z 424 → 190). Typically chromatograms of a standards at the limit of quantification of roflumilast and the metabolite (LLOQ: 0.1 ng/mL) reveal reproducible peaks for the analytes, roflumilast and the metabolite with acceptable assay performances for regulated, routine bioanalysis (Tables 1 and 2). Chromatograms of extracted plasma samples 'spiked' with the penta deuterated standards only show no 'cross-talk' to the traces of the undeuterated analytes (roflumilast and roflumilast N-oxide) at their retention times (1.17 and 0.9 min) and thus proving an adequate isotopic purity of the internal standards.

The detector response for roflumilast and the N-oxide metabolite was linear over the range from 0.1 to 50 ng/mL. The limit of detection was assessed after injection of at least 10 standard samples at concentrations near the lower limit of quantification (LLOQ = 0.1 ng/mL) and subsequent extrapolation to a signal-to-noise ratio of S/N = 3. Accordingly, a limit of detection of approximately 0.06 ng/mL was calculated for both analytes.

Typical calibration curves for roflumilast during a clinical pharmacokinetic study comprised a mean slope of 6.1154 [$n = 11$; relative standard deviation (RSD) = 0.1711] with a mean intercept of −0.0049 (RSD = 0.0035) and a correlation coefficient (r) of 0.999.

Typical calibration curves for roflumilast N-oxide during a clinical pharmacokinetic study comprised a mean slope of 3.9336 [$n = 11$; RSD = 0.0486] with a mean intercept of −0.0032 (RSD = 0.0029) and a correlation coefficient (r) of 0.999.

In order to evaluate the inter-assay performance during method validation of the analytical method we prepared and analysed standard curves on three subsequent days with 24 QC samples with each standard curve in duplicate. Four levels of QC samples were also prepared in duplicate: six at the LLOQ (0.1 ng/mL), six towards the lower quartile (0.3 ng/mL), six in the middle (7.5 ng/mL) and six towards the upper quartile of the calibration curve. The intra-assay performance, analysed on one day of analysis (i.e. within one analytical run) was assessed to ensure that the results were acceptable, and could be used for pharmacokinetic analysis.

The mean inter-assay precision (RSD) for the standards of roflumilast in plasma was ±3.30% with a mean accuracy of 100%, and for the QC samples ±5.14% with a mean accuracy of 98.95% (Table 1). The inter-assay precision (RSD) for the standards of the metabolite in plasma was ±3.93 with a mean accuracy of 99.1%, and for the QC samples ±5.14% with a mean accuracy of 99.7% (Table 2). The mean intra-assay precision (RSD) for the QCs of roflumilast in plasma was ±3.91% with a mean accuracy of 96.7% (Table 3). The mean intra-assay precision (RSD) for the QCs of the metabolite in plasma was ±4.46% with a mean accuracy of 98.8% (Table 4). Therefore, the overall inter- and intra-assay performances during method validation for the standards and QC samples were more than satisfactory for modern routine HPLC–MS/MS bioanalysis.

The reported method can also be applied for both the analysis of roflumilast in plasma from other species (i.e. rat, dog, mouse, rabbit and monkey) and in urine samples. Achieved assay performances for the analysis of roflumilast and roflumilast N-oxide in plasma matrices of other mentioned species are similar to the assay performances in human plasma (results not shown). All mass spectrometric and chromatographic settings as well as the sample work-up procedure are alike to the roflumilast analysis in human plasma.

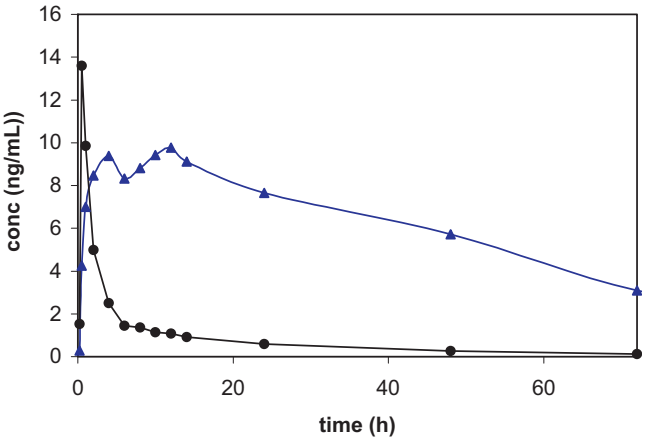

Fig. 8. Plasma concentration versus time profile of roflumilast [(I), ●] and the major metabolite, roflumilast N-oxide [(II), ▲], after oral administration of a single dose of 0.5 mg roflumilast to a healthy male volunteer.

4. Conclusion

In conclusion, a fast, sensitive, selective and robust assay with tandem mass spectrometric detection has been developed. The results of the assay performances clearly demonstrate that an assay for roflumilast and roflumilast N-oxide with a limit of quantification at 0.1 ng/mL could be established utilising semi-automated liquid/liquid extraction and parallel chromatography in a 'dual column mode' linked to a tandem mass spectrometer in the selected reaction monitoring mode. This method has been employed successfully for the determination of the pharmacokinetics of the phosphodiesterase 4 (PDE4) inhibitors, roflumilast and roflumilast N-oxide, in many preclinical and clinical pharmacokinetic and toxicokinetic studies in human plasma and serum.

In Fig. 8 an example of a concentration versus time profile is outlined in which a male healthy volunteer received a single oral dose of 0.5 mg via oral administration. Concentrations of roflumilast (I) and roflumilast N-oxide (II) were measured up to 96 h after administration of roflumilast. It could be demonstrated that maximum plasma levels (C_{max}) for roflumilast and roflumilast N-oxide were achieved at approximately already at 0.5–1, and at 4 h, respectively, after single oral administration of 0.5 mg of roflumilast (within occurring inter-, and intra-individual variabilities in plasma concentrations).

In summary, HPLC–MS/MS in combination with the pneumatically assisted electrospray has once again demonstrated itself to be an excellent and universal tool in today's bioanalysis in supporting pharmacokinetic studies of non-volatile, low molecular mass compounds in particular when sufficient sensitivity and selectivity of classical HPLC assays with ultraviolet or fluorescence detection is lacking.

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References

[1] J.M. Antono, P. Vermeire, J. Vestbo, J. Sunyer, Eur. Respir. J 17 (5) (2001) 982.
[2] G. Viegi, A. Scognamiglio, S. Baldacci, F. Pistelli, L. Carrozzi, Respiration 68 (1) (2001) 4.
[3] P.G. Woodruff, J.V. Fahy, JAMA 286 (4) (2001) 395.
[4] D.S. Bundschuh, M. Eltze, J. Barsig, L. Wollin, A. Hatzelmann, R. Beume, J. Pharmacol. Exp. Ther. 297 (1) (2001) 280.
[5] A. Hatzelmann, C. Schudt, J. Pharmacol. Exp. Ther. 297 (1) (2001) 267.
[6] A. Hatzelmann, E.J. Morcillo, G. Lungarella, S. Adnot, S. Sanjar, R. Beume, C. Schudt, H. Tenor, Pulm. Pharmacol. Ther. 23 (2010) 235.
[7] R.B. Cole (Ed.), Electrospray Ionization Mass Spectrometry Fundamentals Instrumentation and Applications, John Wiley & Sons, Inc., New York, USA, 1997.
[8] N. Mano, J. Goto, Anal. Sci. 19 (2003) 3.
[9] L.Y.T. Li, D.A. Campbell, P.K. Bennett, J. Henion, Anal. Chem. 68 (1996) 3397.
[10] J.P. Allanson, R.A. Biddlecombe, A.E. Jones, S. Pleasance, Rapid Commun. Mass Spectrom. 10 (1996) 811.
[11] C.Y. Yang, Y. Wang, M. Splendore, R.A. Thakur, ASMS Conference, Montreal, Canada, 2003.
[12] M.A. Quilliam, B.A. Scott, K.W.M. Siu, Rapid Commun. Mass Spectrom. 3 (1989) 145.
[13] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (5) (1998) 882.
[14] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019.
[15] B.K. Matuszewski, J. Chromatogr. B 830 (2006) 293.